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# *In Vitro* Cytotoxicity of a Novel Platinum(II) Coordination Complex Containing Diaminocyclohexane

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**Abstract** – We have synthesized a novel platinum(II) coordination complex containing trans-*l*-1,2-diaminocyclohexane (DACH) as a carrier ligand and 1,2-dichloroethane (DCE) as a leaving group. A new series of [Pt(trans-*l*-DACH)(DCE)](PC) was evaluated for its cytotoxic activity on MKN-45 human gastric adenocarcinoma cells and normal primary cultured kidney cells. The new platinum complex has demonstrated high efficacy in the cytotoxicity against MKN-45/P, MKN-45/ADM and MKN-45/CDDP cell-lines. The cytotoxicity of PC against rabbit proximal renal tubular cells, human renal cortical cells and human renal cortical tissues, determined by MTT assay, the [$^3$H]-thymidine uptake and glucose consumption tests, was found to be quite less than those of cisplatin. Based on these results, this novel platinum(II) coordination complex appears to be better for improving antitumor activities with low nephrotoxicity and is a valuable lead in the development of new, clinically available anticancer chemotherapeutic agents.

**Key words** Selective cytotoxicity, Human gastric adenocarcinoma cells, Nephrotoxicity, Platinum coordination complex

The introduction of the square-planar complex cisplatin into the clinical treatment of cancer has resulted in the excellent response rates for some tumor types, especially testicular and ovarian cancers (Rosenberg *et al.*, 1969). While the unfavorable nephrotoxicity profile of cisplatin has been overcome by the development of the second-generation agent, carboplatin, there remains an unquestionable need for additional platinum(II)coordination complexes which have more favorable therapeutic indices and circumvent resistance (Calvert *et al.*, 1985; Harrap *et al.*, 1980). As with other cancer chemotherapeutic agents, cellular resistance to the clinically used platinum agents, cisplatin and carboplatin, represents a major clinical limitation to the efficacy (Einhorn, 1990; Mangioni *et al.*, 1989). It is known that renal cortical accumulation of cisplatin causes necrosis of the proximal tubule and late development of internal cysts (Litterest *et al.*, 1981). The major toxicological limitation is its dose-related accumulative and irreversible side effects of severe nephrotoxicity, nausea and vomiting (Krakoff, 1979).

The antitumor activity of platinum complexes containing 1,2-diaminocyclohexane (DACH) as a carrier ligand was investigated by Connors *et al.*, 1972) and Gale *et al.*, 1974). Kidani (1985) synthesized Pt(oxaloto) (trans-*l*-DACH) [*l*-OPH] using oxalic acid with selected trans-*l*-DACH among trans-*l*-, trans-*d*- and cis- isomers.

Consequently, there is much interest in obtaining agents that have less toxicity and more favorable anticancer chemotherapeutic indices. To accomplish this aim, we have synthesized a new platinum(II) coordination complex containing trans-*l*-DACH as a carrier ligand and 1,2-dichloroethane (DCE) as a leaving group.

In the present study we evaluated *in vitro* antitumor activity of the new platinum complex on the various human gastric adenocarcinoma cell lines and its cytotoxicity on rabbit proximal tubular cells, human renal cortical cells and on histocultured human renal cortical tissues.

## MATERIALS AND METHODS

### Materials

Dulbecco's Modified Eagle's (DMEM), Ham's Nutrient Mixture F12 (F12) and RPMI-1640 media were purchased from GIBCO (Grand Island, NY). Cisplatin, hormones, transferrin, and other chemicals were purchased from Sigma

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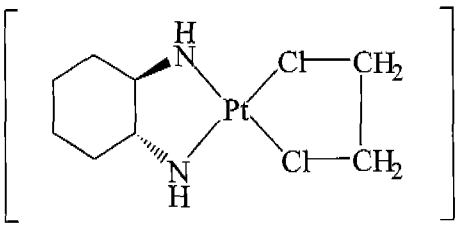

**Fig. 1.** Structure of a new platinum (II) coordination complex. (1,2-Dichloroethane)-trans-*l*-1,2-diaminocyclohexaneplatinum (II) {Pt (II)(trans-*l*-DACH)(DCE)]. DCE : 1,2-Dichloroethane, DACH : 1,2-Diaminocyclohexane

Chemical Co. (St. Louis, MO). Powdered medium, EDTA-trypsin and soybean trypsin inhibitor were obtained from Life Technologies (Grand Island, NY). Class IV collagenase was obtained from Worthington Co. (Freedhold, NY). Iron oxide was prepared by the method described by Cook and Pickering (1958). Stock solutions of iron oxide in 0.9% NaCl were sterilized in an autoclave and diluted with phosphate-buffered saline (PBS) prior to the application. The novel platinum coordination complex, Pt(II)(trans- *l* -DACH)(DCE)[PC](Fig. 1); the PC utilized was synthesized in the Department of Pharmacochemistry, Kyung Hee University College of Pharmacy. This agent was dissolved in sterilized cell culture media prior to usage.

## Cell cultures

### Primary rabbit kidney proximal tubular cells

Each growth study was conducted with an individual primary culture set derived from purified proximal tubules obtained from a single rabbit kidney. Individual kidneys were obtained from male New Zealand White rabbits (2~2.5 kg). Primary cultures were initiated from purified rabbit kidney proximal tubules by a modification of the method described by Chung *et al.*, (1982). The cultures were maintained in a humidified 5% $CO_2$/95% air environment at 37 °C. The purified rabbit kidney proximal tubules attached to the culture dish within a day of plating. The medium was replaced the day after plating so as remove unattached tubules. The medium was replaced every two days thereafter. During the initial 4 days, cell outgrowth occurred from the attached tubules in culture.

### Primary human kidney cortical cells

Normal human kidney tissues were obtained at the time of radical nephrectomy in patients with renal cell carcinoma. Human kidney cortical tissues were washed 3 or 4 times with DME/F12 medium (1 : 1 ratio) supplemented with penicillin G/streptomycin. A single-cell suspension was obtained by mechanical disaggregation with a sterilized surgical knife and subsequent incubation with collagenase (0.124 mg/ml) and trypsin inhibitor (2.5 mg/ml) for 2 minutes. The process was stopped by centrifugation (1000 rpm for 5 minutes) and the particles of the kidney cortical tissues were suspended in DME/F12 medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone ($5 \times 10^{-8}$ M), triiodothyronine (5 μg/ml), prostaglandin $E_1$ ($5 \times 10^{-8}$ M) and 1% fetal bovine serum (FBS) (GIBCO, Grand Island, NY). These suspended cells were seeded in a culture dish and kept in an incubator at 37°C with a highly humidified 5%$CO_2$/95% air. After 2 weeks of incubation, the cells were confluent and used for experiments (Jung *et al.*, 1998).

### Cancer cell lines

Human gastric adenocarcinoma cell lines (MKN-45/P, MKN-45/ADM and MKN-45/CDDP) were obtained from Cell-Biology Lab. of Atomic Power Hospital, Seoul, Korea. MKN-45 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 20 mM sodium bicarbonate, 15 mM HEPES, 92 units/ml penicillin G and 200 μg/ml streptomycin. All the cells were incubated in humidified 5% $CO_2$/95% air at 37 °C.

### Histoculture

Normal kidney tissues, identified by frozen section at the time of radical nephrectomy, were transported in a container to the laboratory which is near the operating room. The normal human kidney tissues were divided into 2~3 mm diameter pieces and five pieces were placed on the top of each previously hydrated Spongostan gel (1 × 1 × 1 cm) (Health Design Industries, Rochester, NY). One gel was put in each well of a six-well plate. 3 ml of MEM supplemented with 10 % FBS, 50 μg/ml gentamicin and 1 μg/ml cefotaxime were added to each well of the plate. The final volume of the medium was sufficient to reach the upper gel surface without immersing it. Culture plates were covered and maintained in humidified 5% $CO_2$ /95% air atmosphere at 37 °C. The histoculture was incubated in medium containing 50 μM of newly synthesized platinum (II) coordination complex and cisplatin, and the specimens were washed with PBS and transferred to fresh medium (Freeman & Hoffman, 1986; Chang *et al.*, 1992).

## *In vitro* antitumor activity

Cancer cell lines were cultured in the growth media for each cell line in an incubator with a highly humidified 5%

$CO_2$/95% air atmosphere at 37 °C. After 3 days of incubation, all cell lines were dissociated with 0.025% trypsin-EDTA for dispersal and centrifuged at 1,000 rpm for 5 minutes. The pellets were suspended with fresh medium. Individual wells of a 96-well tissue culture microtiter plate were inoculated with 0.1 ml of the appropriate medium containing $10^5$ cells. New platinum (II) coordination complex and cisplatin were added in various concentrations. After 48 hr of incubation, 0.05 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well. After 4 hr of exposure, 0.05 ml of DMSO was added to each well and absorption rates at 630 nm were measured using an ELISA reader (Shimoyama *et al.*, 1989).

### Cytotoxicity on normal kidney cells

The confluent primary rabbit kidney proximal tubular and human kidney cortical cells were disaggregated by using 0.02 % EDTA in 0.05% trypsin. Single-cell suspension was then produced by centrifugation (1000 rpm, 10 minutes) and resuspension in DME/F12 medium. This suspension was seeded at a cell density of $10^5$ cells per well on a 96-well plate, with 100 μl of medium per well. Drugs were added in various concentrations (final concentrations : 5, 25, 50, 250 and 500 μM) and the cell cultures were incubated for 48 hr in an incubator a highly humidified atmosphere of 5% $CO_2$/95% air at 37°C. Thereafter, 50 μl of the medium containing MTT (5 mg/ml) was removed and the wells were washed with PBS, and then 50 μl of DMSO was added to each well to solubilize the precipitates. Then the plates were transferred to an ELISA reader to measure the absorbance of the extracted dye at 630 nm. All experiments were performed at least 3 times, with 6 wells for each concentration of the new platinum (II) coordination complex and cisplatin (Mossman, 1973).

### Thymidine uptake

Primary cultured proximal tubular and cortical cells were seeded in each well of a 24-well plate at a cell density of $10^5$ cells per well. After 1 hr of incubation, platinum (II) coordination complexes were added, and the cells were incubated for 48 hr in a humidified incubator containing an atmosphere of 5% $CO_2$/95% air at 37 °C. Thereafter, [$^3$H]-thymidine (1 μCi/ml specific radioactivity) was added to each well, and cells were incubated for 24 hr in the same humidified incubator.

After trypsin-EDTA treatment, all cells were collected and washed twice with 10% TCA and phosphate buffer. The cells were then solubilized with 0.5 M NaOH for 2 hr at 37 °C. The cells were neutralized with 0.5 M HCl and a scintillating cocktail was added (Scint-AXF, Packard, CT). Then the amount of radioactivity uptake by the cells was determined in a β-counter (Beckman, LS 5000 TD).

### Glucose consumption by histocultured specimens.

This study was performed essentially as described by Chang *et al.* (1992). In brief, 50 μl of culture medium was removed every 24 hr for determination of medium glucose content in triplicate using the HK 20 assay kit from Sigma (St. Louis, MO). Measurements were made by monitoring the change in optical density at 340 nm due to the reduction of NAD catalyzed by hexokinase with the glucose content of the medium plotted in semilog form against time using the Sigma plot program (Janel Scientific, Corte, Madera, CA). A simple exponential model of glucose consumption was then fitted to the data with the Systat program (Systat., Inc, Evanston, IL). The half-life of glucose was calculated from the slope parameter of this model using the equation $t = 0.693/s$, where s = slope of the best fit linear regression line or the natural log of the glucose concentration plotted against time. The glucose content of the medium was measured daily for 3 days. The log values over 3 days were plotted against time and the slope of the best-fit line was taken as a glucose consumption rate during the 3-day measurement period (one period).

## RESULTS

### Selective cytotoxicity

The sensitivity of the MKN-45 human gastric cancer cell-lines to the 2 platinum-containing compounds are shown in Table 1. Drug concentration ($CC_{50}$), cytotoxic index by 50%,

**Table 1.** *In vitro* cytotoxicities of Pt (II) complexes on MKN-45 human gastric adenocarcinoma cell lines

| Compounds | $CC_{50}$(M) | | |
|---|---|---|---|
| | MKN-45/P[a)] | MKN-45/ ADM[b)] | MKN-45/ CDDP[c)] |
| Cisplatin | 25.4 ⊥ 3.07[d)] | 23.5 ± 2.83 | 131.6 ± 15.45 |
| PC[e)] | 29.2 + 3.44 | 34.7 ± 5.68 | 60.5 ± 7.20 |

$CC_{50}$ indicates mean cytotoxic concentration with MTT assay,
a) wild type cell,
b) adriamycin-resistant cell,
c) cisplatin-resistant cell,
d) Each value is the mean ± S.E of three experiments,
e) [Pt(II)(trans-*l*-DACH)(DCE)].
*Significantly different from cisplatin-control ($P<0.05$).

**Table 2.** *In vitro* cytotoxicities of platinum complexes on proximal tubular cells of rabbit kidney

| Compounds | $CC_{50}$ (μM) | Ratio[a] |
|---|---|---|
| Cisplatin | 27.5 ± 3.68[b] | - |
| PC[c] | 234.8 ± 31.52 | 8.54 |

a) The ratio is the $CC_{50}$ of the PC divided by that of the cisplatin,
b) Each value is the mean ± S.E of at least three experiments,
c) [Pt(II)(trans-*l*-DACH)(DCE)].
*Significantly different from cisplatin-control(P<0.05).

**Table 3.** *In vitro* cytotoxicities of platinum complexes on cortical cells of human kidney

| Compounds | $CC_{50}$ (μM) | Ratio[a] |
|---|---|---|
| Cisplatin | 24.8 ± 3.08[b] | - |
| PC[c] | 242.5 ± 32.43 | 9.78 |

a) The ratio is the $CC_{50}$ of the PC divided by that of the cisplatin,
b) Each value is the mean S.E of at least three experiments,
c) [Pt(II)(trans-*l*-DACH)](DCE).
*Significantly different from cisplatin-control (P<0.05).

was determined by MTT assay after treatment with the compounds for 48 hr. $CC_{50}$ of cisplatin for the MKN-45/P and MKN-45/ADM cells did not differ significantly.

To investigate renal cytotoxicity, primary cultured rabbit and human normal kidney cells were incubated with various concentrations of cisplatin and the new compound for 48 hr. The cytotoxicities of PC on rabbit kidney proximal tubular cells and human kidney cortical cells were markedly lower, by about 9- and 10-fold, compared with cisplatin, respectively (Table 2 and 3).

### Effect on [$^3$H]-thymidine uptake by normal kidney cells

To evaluate the nephrotoxicity of the new Pt (II) complex, we measured [$^3$H]-thymidine uptake by rabbit proximal tubular cells and human renal cortical cells.

#### Rabbit renal proximal tubular cells

The cytotoxicities of cisplatin and PC against primary cultured rabbit renal proximal tubular cells are shown in Table 2. PC ($CC_{50}$ : 234.8 μM) showed a lower level cytotoxicity compared to cisplatin. In addition to the MTT assay technique, cytotoxicity was determined using [$^3$H] thymidine uptake assay technique. Results are shown in Table 4. At a concentration of $5 \times 10^{-5}$ M, cells treated with PC showed a [$^3$H] thymidine uptake of 55.1%, whereas those treated with cisplatin showed a value of 5.5%. These results indicate that the cytotoxicity of PC was significantly less than that of cisplatin, and the [$^3$H]-thymidine uptake assay is more sensitive than the MTT assay.

**Table 4.** Effect of platinum (II) coordination complexes on $^3$H-thymidine incorporation into primary cultured proximal tubular cells of rabbit-kidney

| Compounds | [$^3$H]-thymidine uptake | uptake rate (%) |
|---|---|---|
| Control | 572.5 ± 68.36 | 100.0 |
| Cisplatin | 31.7 ± 5.41 | 5.5 |
| PC | 315.2 ± 39.05 | 55.1 |

Concentartion of platinum (II) coordination complexes in culture medium; $5 \times 10^{-5}$ M, PC : [Pt (II)(trans-*l*-DACH)(DCE)], Values are means ± S.E. All the incorporations were determined in triplicate.

**Table 5.** Effect of platinum (II) coordination complexes on $^3$H-thymidine incorporation into primary cultured renal cortical cells of human-kidney

| Compounds | [$^3$H]-thymidine uptake | uptake rate(%) |
|---|---|---|
| Control | 590.3 ± 71.50 | 100.0 |
| Cisplatin | 38.6 ± 6.24 | 6.5 |
| PC | 351.7 ± 41.83 | 59.6 |

Concentration of platinum (II) coordination complexes in culture medium; $5 \times 10^{-5}$M, PC : [Pt (II) (trans-*l*-DACH)(DCE)], Values are meansS.E. All the incorporations were determined in triplicate.

#### Human renal cortical cells

PC showed significantly lower cytotoxicity ($CC_{50}$ : 242.5 μM) than cisplatin ($CC_{50}$ : 24.8 μM) (Table 3). Table 5 shows that [$^3$H] thymidine incorporation is more significantly inhibited by cisplatin (59.6%) than by PC (6.5%)in human renal cortical cells.

### Effect on glucose consumption by human kidney tissues

The glucose consumption rate was calculated as the half-life of the glucose in medium which may vary with the drug tested and with the drug concentration (Table 6). In measuring the glucose consumption, one period was defined as more than 3 measurements per day in a 4-week culturing period of human renal cortical tissue. The glucose half-life before adding PC was approximately 60~64 hr and did not show any significant increase for 4 periods, and then increased to over 72 hr from the fifth period up to the 7 th period. However, the effect of PC was less marked than that of cisplatin. The half-life of the medium glucose content was longer in cisplatin-treated cultures than in those treated with PC. Cultures treated

**Table 6.** Change of glucose half-life on histocultured renal cortical tissue specimens that were treated with $10^{-4}$ cisplatin and $10^{-4}$ M experiment drug (PC) for 72 hr

| Compounds | Glucose Half-Life (hours, M ± S.E) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7(period) |
| Cisp | 63.2 ± 8.14 | 61.5 ± 7.35 | 64.8 ± 8.50 | 338.5 ± 41.23 | 377.1 ± 43.20 | 332.0 ± 39.74 | 291.8 ± 31.74 |
| PC | 60.7 ± 8.42 | 62.3 ± 7.70 | 61.6 ± 7.25 | 60.8 ± 8.45** | 66.3 ± 7.94** | 70.4 ± 9.17* | 71.5 ± 8.80* |

One period constitutes 3 days, All experimental histocultures had their own control that was treatments three period, Glucose half-life on control specimen was very steady, Cisp : Cisplatin, PC : [Pt(trans-*l*-DACH)(DCE)]
*Significantly different from cisplatin-control ($P<0.05$, $P<0.01$)

with PC showed a slight increase in glucose half-life compared to the control. In the case of cisplatin the glucose half-life was lengthened after treatment compared with the pre-treatment control. The specimens treated with $10^{-4}$ M of cisplatin still exhibited prolonged glucose half-life in the post-treatment period.

## DISCUSSION

Since Rosenberg *et al.*, (1969) first described the antitumor activity of cisplatin, it has become an important drug in the treatment of selected human malignant tumors. However, its clinical uses are limited by its dose-related renal toxicity. While the unfavorable nephrotoxicity has been overcome by the development of the second-generation agent, carboplatin, there remains an unquestionable need for further improved platinum-containing compounds which have more favorable therapeutic indices and circumvent resistance.

The advanced knowledge of structure-activity relationship of platinum (II) complexes has clarified that the carrier ligands and its leaving groups are essential for their *in vivo* antitumor activities. The contribution of the carrier ligand may be related to the potency and spectrum of antitumor activity, and that of the leaving group may be related to the dissociation rate from the platinum complex. One of the structural modifications that is widely accepted as having resulted in an increased therapeutic index is the attachment of DACH (Clear and Hoeschele, 1973; Connors *et al.*, 1972; Gale *et al.*, 1974; Jung *et al.*, 1998; Ridgway *et al.*, 1977). Several forms of DACH compounds, such as cis-DACH, trans-*l*-DACH, and trans-*d*-DACH, exist. Among these DACH derivatives, trans-*l*-DACH has been known to have more significant antitumor activity (Inagaki and Kidani, 1986). Moreover, it is essential to consider that the leaving group is an important factor influencing the activity of the platinum coordination complexes. The platinum (II) coordination complexes appear to penetrate cell membranes by diffusion, and then the leaving group is displaced directly by hydrolysis with nucleic acid groups. These studies indicated that the dissociation of the leaving groups is an important factor in antitumor and toxic activities. However, when the rate of dissociation is much higher, it causes toxic effects since the platinum complexes react with normal protein instead of DNA in cancer cells. On the other hand, when the dissociation rate is too low, it is excreted into the extracellular compartment before showing any antitumor activity.

The mechanism of the nephrotoxicity induced by platinum (II) coordination complexes is not completely understood. To date, investigators have demonstrated that cytotoxicity induced by a variety of platinum (II) coordination complexes may be attributed at least in part to the inhibition of blood flow in the kidney or the depletion of intracellular glutathione (Meijer *et al.*, 1982). Dobyan *et al.*, (1980) have reported that the site-specific injury was in the pars recta (S3) segment of the proximal tubules. Gonzalez-Vitale *et al.*, (1980) noted that the distal tubule was the most consistently damaged region in the human kidney. Furthermore, a number of investigators (Jones *et al.*, 1986) suggested that both the proximal and distal tubules have been damaged. The nephrotoxicity of cisplatin found in this study is very similar to that reported previously, in terms of both the histopathology and the effects on various measures of renal function (Dobyan *et al.*, 1980; Jones *et al.*, 1986).

The new platinum (II) coordination complex produced and used in this study has generally been screened for antitumor activity and nephrotoxicity using human gastric abeno carcinoma cell lines and normal human and rabbit kidney cells, respectively. The new synthetic platinum (II) coordination complex, PC, exhibited significant *in vitro* antitumor activity against human gastric adenocarcinoma cell lines.

A criteria for *in vitro* antitumor activity is generally expressed in the cytotoxicity index and is accepted as possible antitumor

drugs. PC showed antitumor activity comparable to cisplatin. The comparison of antitumor activities of PC and cisplatin exhibited similarity in levels of activity against MKN-45 human gastric cancer cell lines. Since the only difference in chemical structure between PC and cisplatin is the "DACH-isomer", it can be inferred that the dissociation times or rates are different after absorption of the compounds in the cells due to the different leaving groups.

In this study, PC was less cytotoxic than cisplatin in renal tissues. It is conceivable that the modifications of the carrier ligand as a DACH and the leaving group as a DCE derived from cisplatin significantly decreased nephrotoxicity. Mortine and Borch (1998) reported that the LLC-$PK_1$ (pig proximal tubule epithelial cell line) was a good model in evaluating the nephrotoxicity induced by cisplatin *in vitro*. My present study using primary cultured cells showed reliable data although the LLC-$PK_1$ cell line was not used.

The appearance of glucose in urine is one of the early signs of proximal tubular dysfunction *in vivo*, and therefore we chose the glucose consumption test as a parameter to assess the nephrotoxicity in human renal cortical tissue. Chang *et al.*, (1992) have reported that histocultured renal cortical tissues evaluated using the glucose consumption test provided a good association for cisplatin toxicity. Furthermore, glucose consumption measurements in histocultured human renal cortical tissues were more sensitive than the thymidine-incorporation endpoint (Chang *et al.*, 1994). The results revealed that the newly developed platinum complex has similar or greater anticancer efficacy compared to cisplatin, especially high concentrations. As mentioned above, however, this new complex has very low nephrotoxicity. Therefore, this new complex may possibly be clinically useful for high-dose chemotherapy, with reduced side effects. Based on these results, this novel platinum complex represents a valuable lead and justifies clinical studies in the development of a new anticancer chemotherapeutic agent capable of improving anticancer efficacy with low toxicity.

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